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# Oxidative stress adaptation in aggressive prostate cancer may be counteracted by the reduction of glutathione reductase

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## ABSTRACT

**Oxidative stress has been associated with prostate cancer development and progression due to an increase of reactive oxygen species (ROS). However, the mechanisms whereby ROS and the antioxidant system participate in cancer progression remain unclear.**

**In order to clarify the influence of oxidative stress in prostate cancer progression, we performed this study in two human prostate cancer cell lines, PC3 and HPV10 (from metastasis and from localized cancer, respectively) and RWPE1 cells derived from normal prostate epithelium. Cells were treated with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and PC3 cells were also treated with diethyl maleate (DEM). The effect on cell growth, viability, mitochondria membrane potential and oxidative stress was analysed. Oxidative stress was evaluated based on ROS production, oxidative lesion of lipids (MDA) and on determination of antioxidants, including enzyme activity of glutathione peroxidase (Gl-Px), glutathione reductase (Gl-Red) and on the quantification of glutathione (GSH), glutathione-s-transferase (GST) and total antioxidant status (TAS).**

**PC3 shows higher ROS production but also the highest GSH levels and Gl-Red activity, possibly contributing to oxidative stress resistance. This is also associated with higher mitochondrial membrane potential, TAS and lower lipid peroxidation. On the other hand, we identified Gl-Red activity reduction as a new strategy in overcoming oxidative stress resistance, by inducing H<sub>2</sub>O<sub>2</sub> cytotoxicity. Therefore these results suggest Gl-Red activity reduction as a new potential therapeutic approach, in prostate cancer.**

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## 1. Introduction

Prostate cancer is still the most frequently diagnosed malignant disease and the second leading cause of cancer-related mortality in men in most Western countries [1,2].

Although the causes of the high incidence of prostate cancer are poorly understood, epidemiological, experimental and clinical studies, suggest that oxidative stress (OS) plays a major role in explaining prostate cancer development and progression [3–12]. OS, defined as an imbalance between reactive oxygen species (ROS) production and antioxidant defences [13,14] has been linked to some prostate cancer risk factors including diet intake [15–18], recurrent inflammation and ageing [19–21].

ROS, generated in vivo, include free radicals and non-radicals. Free radicals are molecules containing unpaired electrons as superoxide anion ( $\cdot\text{O}_2^-$ ), hydroxyl radical ( $\cdot\text{OH}$ ) and peroxide radicals [22]. Non-radicals such as singlet molecular oxygen ( $\text{O}_2$ ), nitrogen oxide ( $\text{NO}_x$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) can easily give rise to oxygen radicals. Namely,  $\text{H}_2\text{O}_2$  leads to  $\cdot\text{OH}$  formation, in the presence of transition metals [22,23]. ROS play an essential role in signal transduction pathways [24], cell cycle progression [25–30], gene transcription [31], cell differentiation [28,32] and death (for

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review see Martindale and Holbrook [33]). However, an increase in ROS production and/or a decrease in antioxidant network may induce to severe OS leading to biomolecules damage such as DNA, proteins and lipids [3–12,34,35]. On the other hand, oxidative damage of DNA is thought to play a critical role in all stages of carcinogenesis [36].

Moreover, the major feature of radiation therapy that is a standard treatment of prostate cancer is based in ROS generation leading to oxidative damage [37]. However, metastatic prostate cancer cells may be resistant to radiotherapy, suggesting that the antioxidant system may play an important role in circumventing radiation cytotoxicity [34], thus, contributing for therapy failure. Therefore new therapeutic approaches related, in part, with OS modulation, have been suggested, namely, by Freitas et al. [38].

The antioxidant network comprises the enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GPx) and glutathione-S-transferase (GST) that play an important role in prostate cancer prevention, protecting cells from genomic damage mediated by carcinogens and ROS generated during inflammation. Other molecules, as vitamins E and C and reduced glutathione (GSH) complement the antioxidant enzymes and are capable of neutralizing ROS [22]. GSH plays a critical role in cellular redox maintenance. GPx catalyses the reduction of peroxides and the formation of oxidized glutathione (GSSG) [7]. GPx uses NADPH and  $H^+$  to reduce the GSSG back to GSH [9]. This paper investigates whether ROS (peroxides) and antioxidant defences contribute to prostate cancer progression and how the OS modulation may be a new prostate cancer therapeutic approach.

## 2. Materials and methods

### 2.1. Cell culture conditions

Human prostate cancer cell lines derived from localized adenocarcinoma, from (HPV10) [39], from bone metastasis (PC3) [40] and from the normal prostate epithelium (RWPE1) [41] were purchased from the American Type Culture Collection (ATCC) and cultured in optimum growth conditions.

RWPE1 and HPV10 cells were grown in keratinocytes medium (Gibco) supplemented with 5 ng/ml of human recombinant epidermal growth factor (rEGF) (Gibco) and 0.05 mg/ml of bovine pituitary extract (BPE) (Invitrogen, formerly Gibco-BRL). PC3 cells were grown in RPMI 1640 medium (Sigma) with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Biocrom) and 2 mM L-glutamine (Sigma). Both medium, were supplemented with 100 U/ml Penicillin, 100 µg/ml Streptomycin and with 5 µg/ml Kanamycin (Sigma).

Cells were maintained in a 95% humidified incubator with 5%  $CO_2$  at 37 °C and were passaged with trypsinization every fourth day. For assays RWPE1 and HPV10 were plated at a density of  $5 \times 10^5$  cells/ml whereas PC3 were seeded at a density of  $3 \times 10^5$  cells/ml. After being cultured for 24 h, the cells were washed once with fresh assay medium and treated for 24–72 h with hydrogen peroxide ( $H_2O_2$ ) (10 nM–500 µM). PC3 were also treated with diethyl maleate (DEM) (Sigma).

### 2.2. Cell proliferation analysis

Cell proliferation was measured by the colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) assay method that quantifies the reduction of the yellow tetrazolium salt to purple formazan crystals by the mitochondria of viable cells [42]. Briefly, untreated and treated cells were washed with PBS (Gibco) that was replaced by MTT (0.5 mg/ml) supplemented with 1 mM  $CaCl_2$  (Sigma). The cells were then incubated at 37 °C for 2 h. Formazan crystals were dissolved with HCl

0.04 M in isopropanol. Absorbance from the resultant coloured solution was measured at 570 nm [38].

### 2.3. Flow cytometry studies

Each 24 h of incubation,  $1 \times 10^6$  of treated cells and corresponding controls, were collected by trypsinization and washed two times in PBS buffer, by centrifugation, for further acquisition and analysis in a FACScalibur (488 and 635 nm), using the Cellquest and Paint-a-gate software (BD Bioscience). Attached cells are considered as viable and were selected for mitochondrial membrane potential (MMP) and ROS analysis. For cell viability and death analysis we also collected the suspension cells.

Specimens were prepared in triplicate and at least 10000 events were collected.

#### 2.3.1. Cell viability and death: detection of apoptosis or necrosis using Annexin-V/propidium iodide incorporation

For identification of cell death by apoptosis or necrosis, suspension and attached cells were collected for the assay. After washed, the collected cells ( $1 \times 10^5$ ) were resuspended in 100 µl of binding buffer (0.025 M  $CaCl_2$ , 1.4 M NaCl, 0.1 M Hepes) containing 5 µl Annexin-V APC and 2 µl propidium iodide 3 µM (PI) (Invitrogen-Molecular Probes). Samples were kept in the dark at room temperature for 15 min, according to manufacturer's instruction [43] and Freitas et al. [38].

#### 2.3.2. Mitochondrial membrane potential (MMP) analysis

In order to detect MMP, the cells were labelled with the fluorescent probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC1) (Cell Technology) according to manufacturer's instructions as previously performed by Freitas et al. [38]. Briefly  $5 \times 10^5$  cells were resuspended in 0.5 ml of  $1 \times$  JC-1 reagent solution and then incubated for 15 min, at 37 °C in a 5%  $CO_2$  chamber. The cells were washed two times with 2 ml  $1 \times$  assay buffer under centrifugation at 1500 rpm, resuspended in 0.5 ml  $1 \times$  assay buffer and were analysed by flow cytometry.

The lipophilic cationic probe JC1, developed by Cossariza et al. [44] is able to selectively enter in the intact mitochondria, forming J-aggregates (J-A), which are associated with a large shift in emission (590 nm). However, in lower polarized mitochondrial membrane, JC1 accumulates in the cytoplasm in the monomeric form (J-M), emitting at 527 nm after excitation at 490 nm. Therefore the ratio J-M/J-A is inversely correlated with MMP.

### 2.4. OS evaluation

OS was evaluated by measuring ROS production, antioxidant capacity and lipid peroxidation.

#### 2.4.1. Reactive oxygen species measurements

We evaluate ROS levels by labelling  $5 \times 10^5$  cells with 5 µM 2,7-dichlorodihydrofluorescein diacetate (DCFH<sub>2</sub>-DA) (Sigma) according to adaptations of previously procedures [38,45–47]. The cells were incubated during 1 h at 37 °C, in the dark, washed two times in 0.5 ml phosphate-buffered saline (PBS) and were collected through centrifugation at 1500 rpm. The cells were then resuspended in 0.5 ml PBS for flow cytometry analyses. This methodology is based on the conversion of (DCFH<sub>2</sub>-DA) in DCFH<sub>2</sub> by intracellular esterases and consequent formation of the highly fluorescent 2,7-dichlorofluorescein (DCF) by ROS. The resultant green fluorescence is proportional to the intracellular level of ROS, upon excitation at 488 nm. Moreover total population was considered for ROS measurements.

### 2.4.2. Antioxidant capacity

**2.4.2.1. Cell lysates preparation.** After 24 h, treated cells were washed two times with PBS, scraped off the flasks and resuspended in 1 ml PBS. Cells were then subjected to three pulses of sonication for 10 s with 1 min intermittent cooling on ice in a Bandelin Sonorex.

Protein concentration was assessed using bicinchonic acid assay kit (Sigma) according to manufacturer's instruction. The lysates had been stored at  $-80^{\circ}\text{C}$  before usage.

**2.4.2.2. Reduced glutathione assay.** Reduced glutathione (GSH) was performed using a kit from Oxisresearch according to manufacturer's instructions. This method is based on the formation of a chromophoric thione that is proportional to GSH concentration at 420 nm [48]. Results are expressed as  $\mu\text{mol}$  of GSH per gram of protein ( $\mu\text{mol/g prot}$ ).

### 2.4.2.3. Antioxidant enzymes determinations.

**2.4.2.3.1. Glutathione peroxidase.** Glutathione peroxidase (GPx) activity was evaluated by spectrophotometry using tert-butylperoxide as a substrate [49], monitoring the formation of oxidized glutathione, through the quantification of the oxidation of NADPH to  $\text{NADP}^{+}$  at 340 nm. Results are expressed in international units of enzyme per gram of protein (U/g prot).

**2.4.2.3.2. Glutathione reductase.** Glutathione reductase (GR) activity was determined using GSSG as a substrate and monitoring its reduction to GSH through quantification of NADPH oxidation at 340 nm [50] in a thermostated spectrophotometer UVIKON 933 UV/Visible, at  $37^{\circ}\text{C}$ . GR activity was expressed in international units of enzyme per gram of protein (U/g prot).

**2.4.2.3.3. Glutathione-S-transferase.** Glutathione-S-transferase (GST) levels, namely from the Pi subgroup (GST-Pi), were quantified by an Enzyme Immuno Assay (EIA) according to manufacturer's instructions (Immunodiagnostick) at 450 nm. This method is based in the competition of GST samples and GST from plate for a rabbit antibody binding. After a washing step, the detection of the bound rabbit antibody is performed by a peroxidase labelled goat antibody anti rabbit (POD-antibody). The amount of converted substrate (TMB) is indirectly proportional to the amount of GST antigen in the sample [51]. The results are expressed as  $\mu\text{mol}$  of GST per gram of protein ( $\mu\text{mol/g prot}$ ).

### 2.4.3. Lipid peroxidation evaluation

Oxidative lesion of lipids was evaluated by the formation of a thiobarbituric acid (TBA) adduct of malondialdehyde (MDA) and then separated by HPLC [52,53]. Cell lysates were boiled during 60 min with TBA and phosphoric acid, then were deproteinized with methanol/NaOH 1 M (10:1) and centrifuged. The supernatant (20  $\mu\text{l}$ ) was injected into a Spherisorb ODS2 5  $\mu\text{m}$  (250  $\times$  4.6 mm) column. Elution was performed with 60% (v/v) potassium phosphate buffer 50 mM, pH 6.8, and 40% (v/v) methanol at a flow rate of 1 ml/min. The TBA-MDA adducts were detected at 532 nm and quantified by extrapolating the area of the peaks from a calibration curve of 1,1,3,3-tetraethoxypropane (TEP) standard solutions. Results are expressed as  $\mu\text{mol}$  of MDA per gram of protein ( $\mu\text{mol/g prot}$ ).

### 2.4.4. Total antioxidant status (TAS) determination

TAS was determined by a chromogenic method (Randox Laboratories Crumham's, North Ireland) with briefly adaptations. This methodology is based on the capacity to inhibit the formation of the ABTS $^{+}$  radical cation (2,2'-azino-di-[3-ethylbenzothiazolin sulphinate]) and detection at 600 nm in a spectrophotometer UVIKON 933-UV/Visible, thermostated and computerized [54]. Results are expressed as  $\mu\text{mol}$  of TAS per gram of protein ( $\mu\text{mol/g prot}$ ).

### 2.5. Statistics

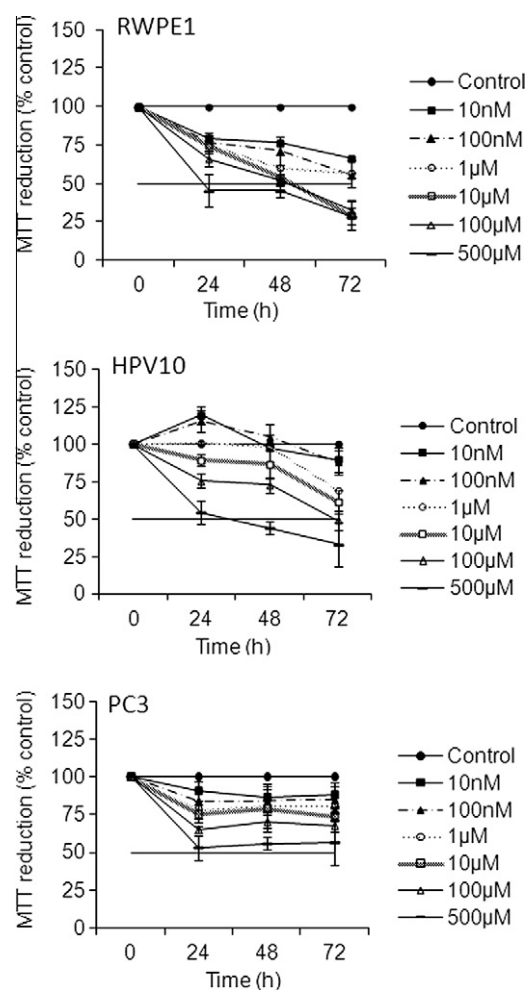
Statistical analyses were carried out using *t*-tests. Significance was assessed for *P* values  $<0.05$ .

## 3. Results

### 3.1. Metastatic prostate cancer cells are more resistant to $\text{H}_2\text{O}_2$ than the other cell lines

#### 3.1.1. Effect of $\text{H}_2\text{O}_2$ on cell growth and viability

Fig. 1 represents the proliferative effect of  $\text{H}_2\text{O}_2$  (10 nM–500  $\mu\text{M}$ ) on a cell line derived from human normal prostate epithelium (RWPE1) and on prostate cancer cells, derived from localized and metastatic carcinoma (HPV10 and PC3, respectively), during 72 h. As we observe  $\text{H}_2\text{O}_2$  induces different effects according to cell type, time of incubation and concentration exposure. Therefore  $\text{H}_2\text{O}_2$  inhibits RWPE1 cell growth for all tested concentrations whereas PC3 seems to maintain a cell proliferation rate above



**Fig. 1.** Dose–response curves. The effect of different  $\text{H}_2\text{O}_2$  concentrations (10 nM–500  $\mu\text{M}$ ) on proliferation of human normal prostate epithelium (RWPE1) and prostate cancer cells, derived from localized and metastatic carcinoma (HPV10 and PC3) are represented. Proliferation was evaluated through the formation of formazan products by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), each 24 h, during 72 h of incubation, as referred in materials and methods. Results are expressed as percentage of MTT reduction relatively to control (cells not treated with  $\text{H}_2\text{O}_2$ ) and correspond to the mean  $\pm$  S.D. of at least three separate experiments.

the half maximal inhibitory concentration (IC<sub>50</sub>). On the other hand we found that low H<sub>2</sub>O<sub>2</sub> concentrations induce an increase in HPV10 cells proliferation.

We observed that the effect of H<sub>2</sub>O<sub>2</sub> on cell proliferation inhibition, namely at IC<sub>50</sub> concentration is associated to cell death mainly by necrosis, in HPV10 and RWPE1 cells. Data not shown, also indicate that lower ROS concentration, namely 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>, induced necrosis in RWPE1 and HPV10. However, PC3 maintain cell viability besides a decrease in cell proliferation (Fig. 2). These results suggest a more efficient adaptation to peroxides in PC3.

### 3.1.2. Influence of H<sub>2</sub>O<sub>2</sub> on MMP

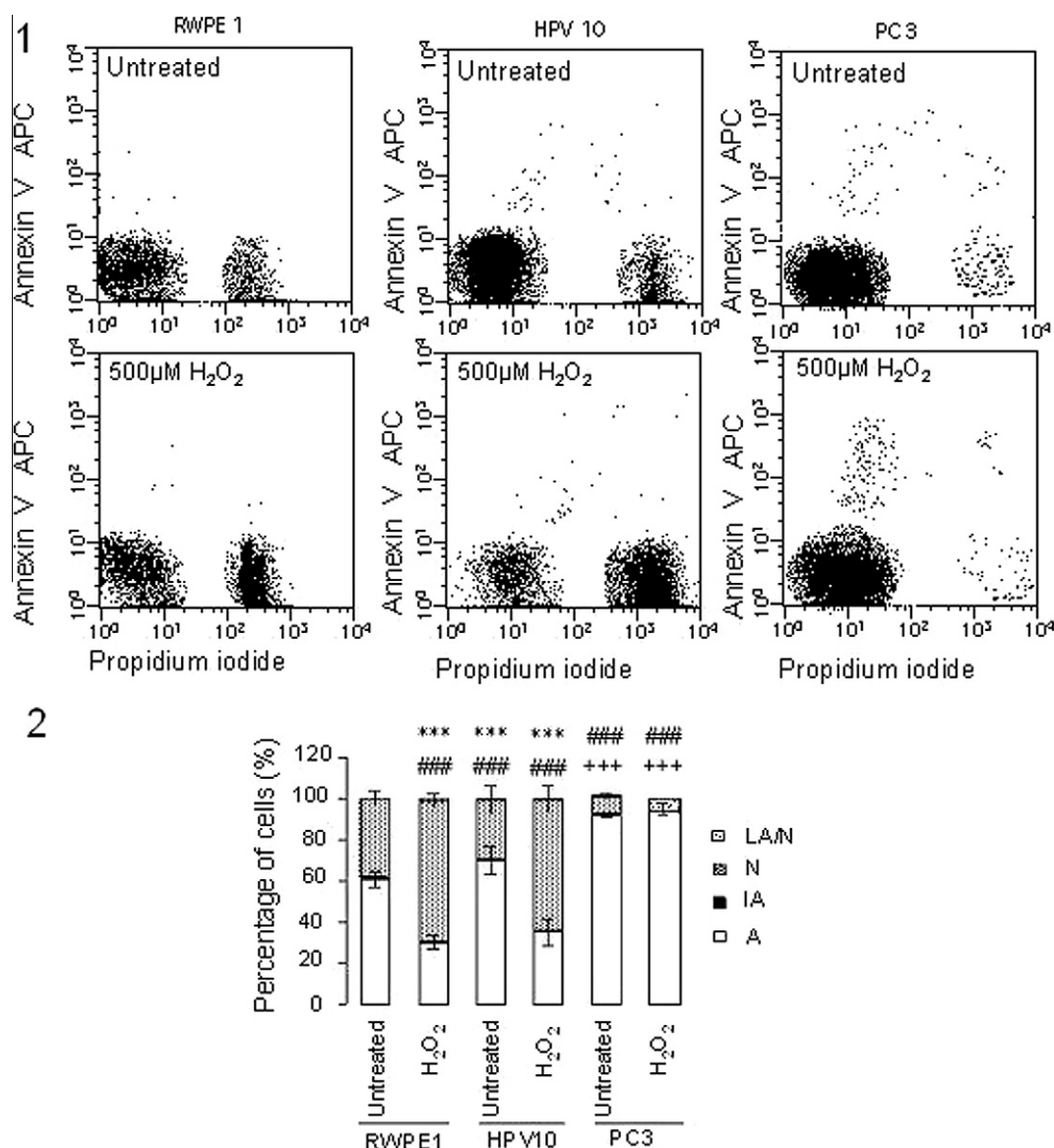
To evaluate the role of mitochondria on prostate cancer progression/metastasis and the effect of H<sub>2</sub>O<sub>2</sub> on MMP we used the JC1 assay. Fig. 3 shows that PC3 cells had the highest basal MMP and RWPE the lowest, which is in line with viability and proliferative results. However, in the presence of H<sub>2</sub>O<sub>2</sub>, we observe a

significant decrease in MMP, in RWPE1 and HPV10 cells, as demonstrated by the increase of monomers/aggregates (M/A) ratio that also agree with viability and proliferative results.

### 3.1.3. Metastatic prostate cancer cells are resistant to ROS (peroxides) by an increase in GSH content and Gl-Red activity

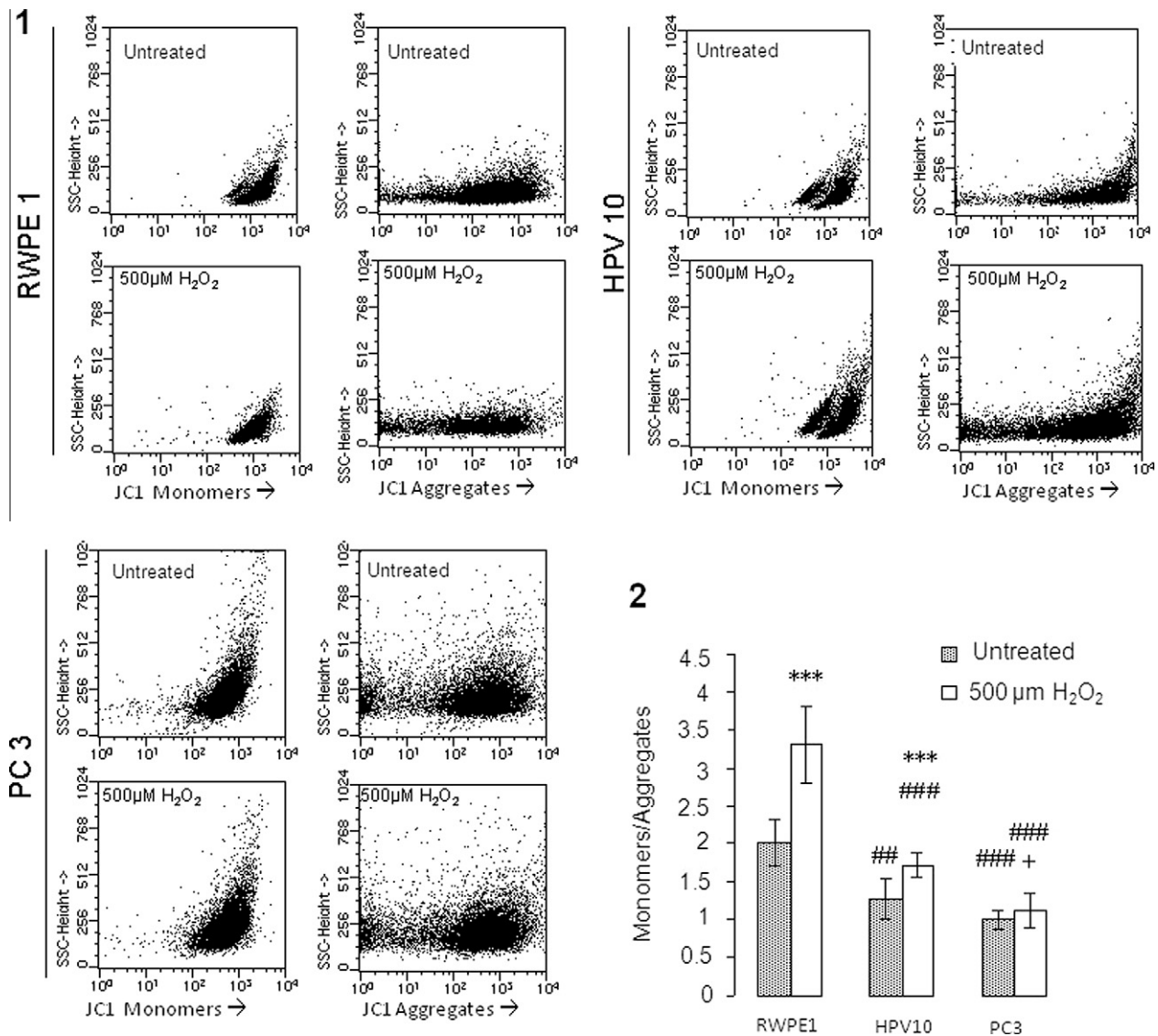
To evaluate the role of H<sub>2</sub>O<sub>2</sub> on ROS (peroxides) production we used the 2,7-dichlorodihydrofluorescein diacetate (DCFH<sub>2</sub>-DA) probe, a dye that fluoresces in the presence of peroxides (H<sub>2</sub>O<sub>2</sub>) [46].

This study shows that prostate cancer cells, particularly the metastatic cells (PC3), exhibit significant higher ROS levels compared with the others (Fig. 4). On the other hand the sensitivity to cytotoxicity induced by ROS (H<sub>2</sub>O<sub>2</sub>) in RWPE1 and HPV10 is confirmed by an increase in lipid peroxidation as observed in Fig. 5. In opposite, we found a decrease in lipid peroxidation and increase of TAS in PC3 (Fig. 5). These results suggest that PC3 cells are resistant



**Fig. 2.** Effect of H<sub>2</sub>O<sub>2</sub> on cell viability and death in RWPE1, HPV10 and PC3 cells. Dot-plot profiles (1) were obtained after the acquisition of 10000 events. Cells were cultured in the absence (untreated) or in the presence of 500  $\mu$ M H<sub>2</sub>O<sub>2</sub> during 24 h. Cells were incubated with Annexin-V/PI as referred in materials and methods. Alive cells (A) exclude propidium iodide and do not bind Annexin-V. Apoptotic cells with intact membranes exclude propidium iodide, externalize phosphatidylserine to the outside of the plasma membrane and therefore bind Annexin-V (IA) emitting fluorescence. Propidium iodide stains nuclear DNA of necrotic (N) and late apoptosis/necrosis cells (LA/N). Results were expressed as the percentage of cells staining with the respective molecular probe (2). The results represent the means  $\pm$  S.D. of at least triplicate determinations. Significantly viability and necrosis differences are considered: \*\*\* $P$  < 0.001 vs untreated samples, ### $P$  < 0.001 for PC3 and HPV10 vs RWPE1 and +++ $P$  < 0.001 for PC3 vs HPV10.





**Fig. 3.** MMP evaluation in RWPE1, HPV10 and PC3 cells. Dot-plot profiles (1) were obtained after the acquisition of 10000 events. RWPE1, HPV10 and PC3 cells were cultured in the absence (untreated) or in the presence of 500  $\mu$ M  $H_2O_2$  for 24 h. The MMP was assessed by flow cytometry labelling the cells with the molecular probes JC1 as described in materials and methods. Results were expressed as the ratio of Monomers/Aggregates (M/A) (which are inversely proportional to MMP) and represent the means  $MMP \pm S.D.$  of at least triplicate determinations (2). Significantly viability differences are considered: \*\*\* $P < 0.001$  vs untreated samples, ### $P < 0.001$  and ## $P < 0.01$  for PC3 and HPV10 vs RWPE1 and \* $P < 0.05$  for PC3 vs HPV10.

to ROS which may contribute to a more aggressive phenotype, related with prostate progression and metastasization. In order to determine the contribution of the antioxidant system in cells adaptation to ROS, we analysed GSH and GST content and Gl-Red and Gl-Px activities, simultaneously in the three cell lines. Results represented in Fig. 6 indicate a significant decrease in GST (Fig. 6D) content and Gl-Px (Fig. 6B) activity in the malignant cells, particularly in PC3, which may be related with higher  $H_2O_2$  levels. On the other hand, PC3 have the highest GSH content and Gl-Red activity that could contribute to resistance to OS (Fig. 6C and A, respectively).

#### 3.1.4. Adaptation to peroxides (ROS) is reverted by DEM

In order to evaluate the modulation of ROS resistance by GSH content, the major thiol in mammalian cells, and Gl-Red activity, PC3 cells were treated with a thiol depleting agent (DEM), during 24 h.

As we can observe in Fig. 7A1 and A2, DEM induces a decrease in GSH content and Gl-Red activity concomitantly with a decrease

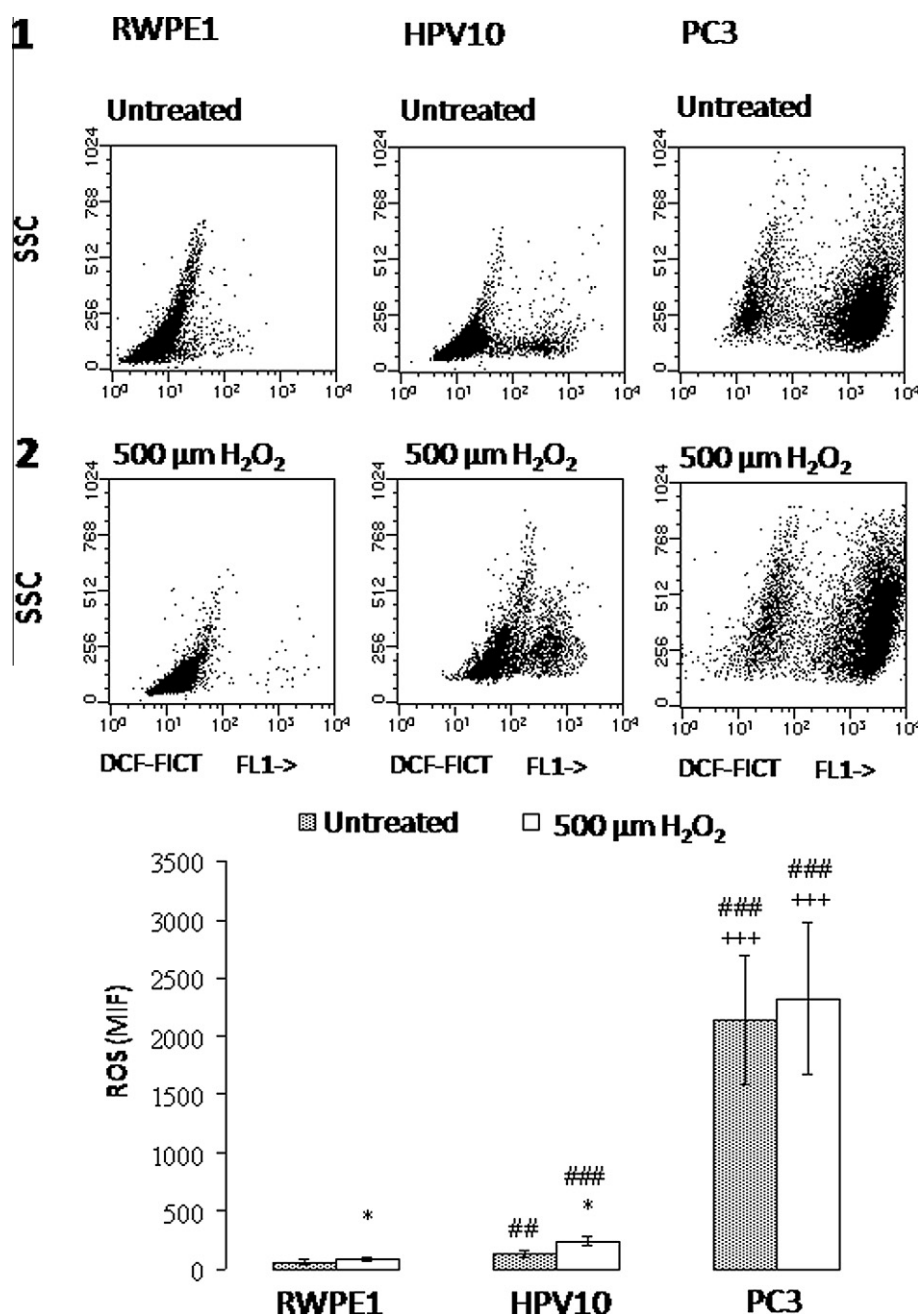
in cell proliferation (Fig. 7B1) and an increase of cytotoxicity mediated by  $H_2O_2$  (ROS) in PC3 cells (Fig. 7C).

In fact, the association of DEM and 500  $\mu$ M  $H_2O_2$  induced a decrease in cell growth (Fig. 7B2) and viability (Fig. 7C), when compared with 500  $\mu$ M  $H_2O_2$  alone. This is accompanied by an increase in cell death mainly by apoptosis and late apoptosis/necrosis after 24 h treatment with DEM (Fig. 7C).

#### 4. Discussion

Development of efficient therapies requires a better understanding of the mechanisms underlying prostate carcinogenesis. Although OS has been associated with prostate cancer development and progression due to an increase of ROS [7,55], the mechanisms whereby ROS and antioxidants may induce cancer progression remain unclear [12]. As a result, greater understanding of OS may be of considerable importance for fighting prostate cancer.

In line with existing literature, we observed that the effect of  $H_2O_2$  on cell proliferation is dose, time and cell type dependent.



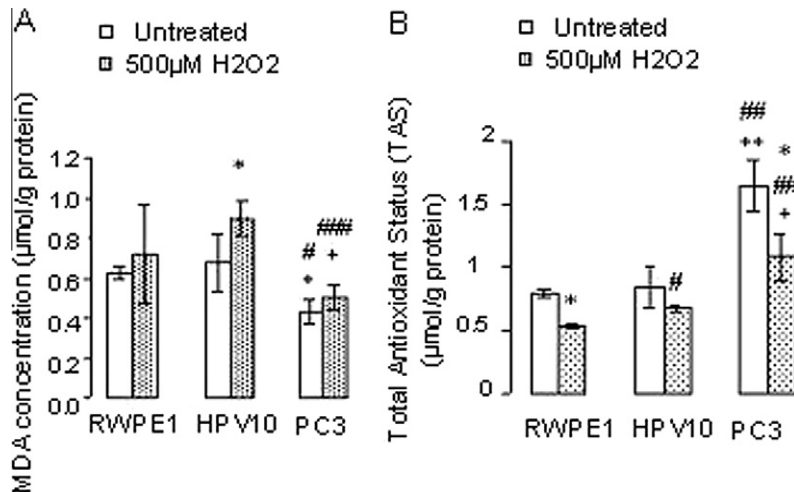
**Fig. 4.** Effect of  $\text{H}_2\text{O}_2$  on ROS production in RWPE1, HPV10 and PC3 cells. Dot-plot profiles (1) were obtained after the acquisition of 10000 events. RWPE1, HPV10 and PC3 cells were cultured in the absence (untreated) or in the presence of  $500 \mu\text{M}$   $\text{H}_2\text{O}_2$  for 24 h. ROS was assessed by flow cytometry labelling the cells with 2,7-dichlorodihydrofluorescein diacetate (DCFH<sub>2</sub>-DA). The results were expressed as the means intensity fluorescence (MIF)  $\pm$  S.D. of at least triplicate determinations (2). Significantly differences are considered: \* $P < 0.05$  vs untreated samples, \*\* $P < 0.01$ , \*\*\* $P < 0.001$  for PC3 and HPV10 vs RWPE1 and +++ $P < 0.001$  for PC3 vs HPV10.

In particular, low levels of  $\text{H}_2\text{O}_2$  (10–100 nM) induced cell growth, in HPV10, derived from localized carcinoma, suggesting that small increments of ROS may be important in progression of prostate cancer. Interestingly, Sikka et al. [7] previously demonstrated that low levels of  $\text{H}_2\text{O}_2$  (30 pM–300 nM), induce cell growth on benign prostate hyperplasia (BPH), characterized by an intense cell proliferation rate and considered as potential precursor of prostate cancer [56]. On the other hand, chronic inflammation of prostate epithelium, due to persistent ROS production, has been associated with increase of OS and DNA damage leading to neoplastic transformation (for a good review of this literature see Nelson et al. [19]). However, an excess of ROS is expected to be harmful and to induce apoptosis in several human tumour cell lines [19]. Here we found that  $500 \mu\text{M}$   $\text{H}_2\text{O}_2$  inhibits cell proliferation in all the

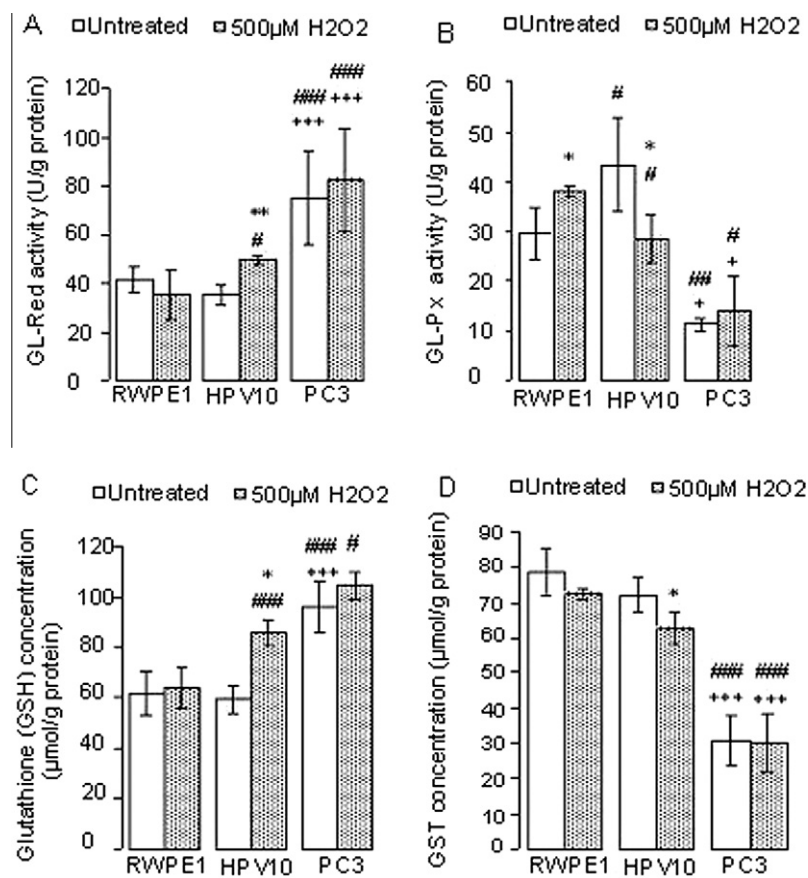
tested cell lines, but the effect was less pronounced in PC3. These differences may be notably observed after 72 h of treatment.

Moreover it was accompanied by cell death, essentially by necrosis, in HPV10 and in the normal epithelium cell line, RWPE1 just after 24 h treatment. Similar results were found by Sikka et al. [7] in BPH cell lines with  $300 \mu\text{M}$   $\text{H}_2\text{O}_2$ . However, higher  $\text{H}_2\text{O}_2$  concentrations did not affect PC3 viability that appears to be more resistant to OS. It would be interesting to evaluate whether  $\text{H}_2\text{O}_2$  causes some cycle phase-specific blockade, since drug-provoked oxidative stress causes cell cycle disruption as well as cell death. This may also be important in cancer treatment.

We observed the highest basal levels of ROS in PC3 (Fig. 4) which may be related to cells resistance to  $\text{H}_2\text{O}_2$  induced cytotoxicity and to a more aggressive phenotype, although others have



**Fig. 5.** Evaluation of lipid peroxidation and TAS in RWPE1, HPV10 and PC3 cells. Cells were treated with 500 μM H<sub>2</sub>O<sub>2</sub> for 24 h as described in materials and methods. Lipid peroxidation (A) was evaluated by MDA determination levels. MDA and TAS (B) levels were detected according with described in material and methods. Results were expressed as the means ± S.D. of at least triplicate determinations. Significant differences are considered: \**P* < 0.05 vs untreated samples, #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 for HPV10 and PC3 vs RWPE1 and \**P* < 0.05, \*\**P* < 0.01 for PC3 vs HPV10.

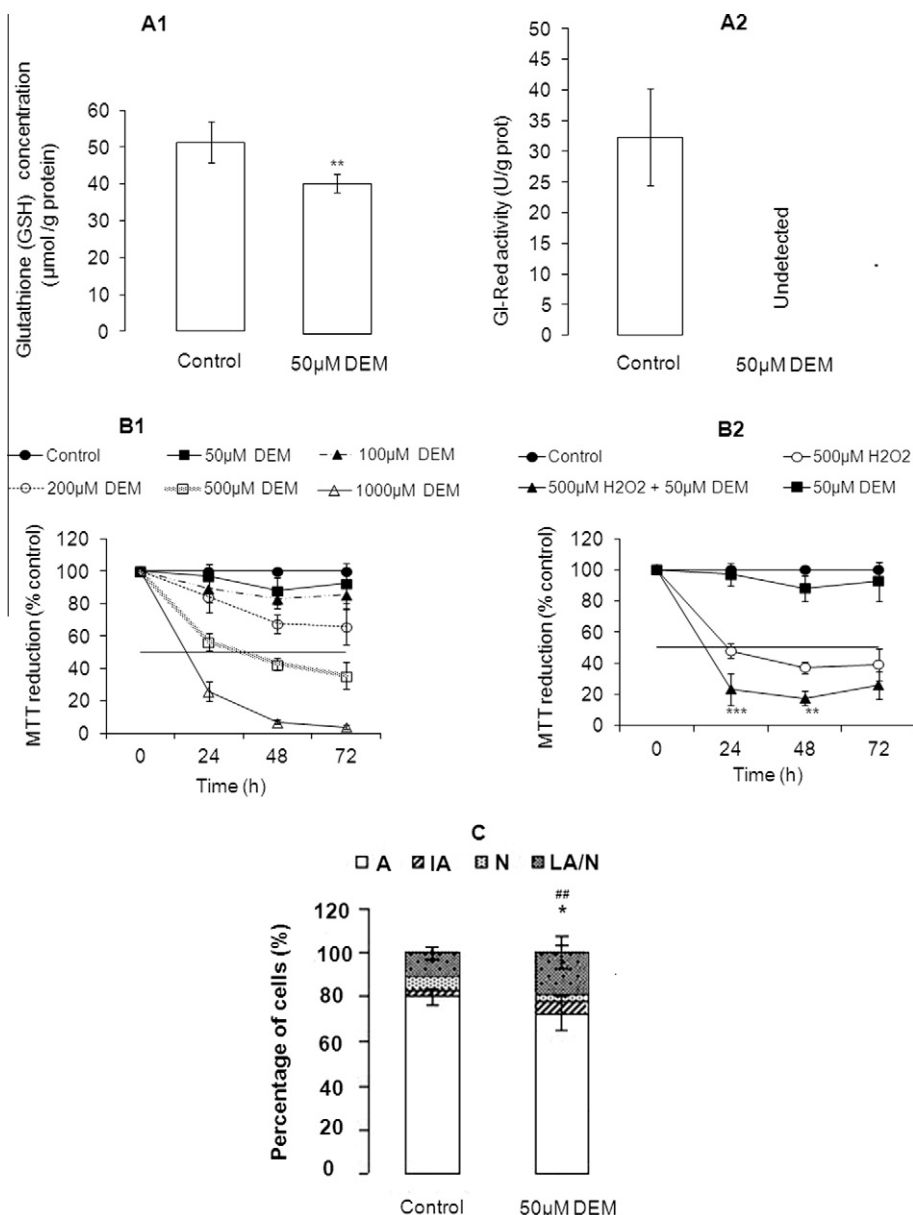


**Fig. 6.** Antioxidant defences in RWPE1, HPV10 and PC3 cells. We evaluate GL-Red (A) and GL-Px (B) activities and GSH (C) and GST (D) content. Cells were treated with 500 μM H<sub>2</sub>O<sub>2</sub> for 24 h as described in materials and methods. Results are expressed as the means ± S.D. of at least triplicate determinations. Significant differences are considered for \**P* < 0.05, \*\**P* < 0.01 vs untreated samples, #*P* < 0.05, ##*P* < 0.01, and ###*P* < 0.001 for HPV10 and PC3 vs RWPE1 and \**P* < 0.05 and \*\*\**P* < 0.001 for PC3 vs HPV10.

found that increase of ROS is related with cell injury, resulting from oxidative damage of biomolecules such as DNA, proteins and lipids [3–12,34,35]. It is also important to realize that tumour progression is based on DNA mutations which promote cancer cells proliferation and surveillance [57]. In part, these mutations may be

acquired as a result of an increase of ROS which require an antioxidant system adaptation [58].

Viability results are in line with MMP detected in cells. In fact, at basal conditions, PC3 cells show the highest MMP whereas RWPE1 cells show the lowest MMP. These results may be related



**Fig. 7.** Effect of DEM on PC3 antioxidant defences – relation with cell proliferation and viability. GSH content (A1) and Gl-Red activity (A2) on PC3 were analysed after treating cells with 50 μM DEM for 24 h. The effect of DEM (50 μM–1000 μM) alone (B1) and combined with 500 μM H<sub>2</sub>O<sub>2</sub> (B2) on PC3 cells growth was evaluated during 72 h. In (C) is represented the influence of DEM on PC3 cell viability and death. Cells were treated with 500 μM H<sub>2</sub>O<sub>2</sub>, in the absence (control) and in the presence of 50 μM DEM for 24 h. Viability was assessed by flow cytometry as previously described. Viable cells are expressed by alive cells (A), initial apoptosis by (IA), necrosis (N) and late by apoptosis/necrosis (LA/N). Results were expressed as the means ± S.D. of at least triplicate determinations. Significant differences are considered for \**P* < 0.05, \*\**P* < 0.01 and \*\*\**P* < 0.005 vs control. Cell viability and death statistical results are indicated for (IA) and (LA/N).

with cell vulnerability to ROS induced cytotoxicity. In the same way, when PC3 were treated with 500 μM H<sub>2</sub>O<sub>2</sub> we did not observe a significant decrease in MMP, in contrast to other cell lines. Based on this evidence we also suggest that variation in MMP is related to cell growth but not in a directly proportional ratio.

In order to better understand the effect of OS in prostate cancer progression we evaluated the levels of ROS and antioxidant defences among the distinct cell lines. We found that ROS levels are lower in RWPE1 and substantially higher in PC3 as we have previously referred. These findings are in agreement with those observed by Kumar et al. [55]. The same authors show that the degree of ROS generation is directly proportional to aggressive phenotype. They also found higher levels of ROS in PC3 compared to RWPE1, although, they did not analyse localized prostate cancer cell lines as performed in our work. Likewise we demonstrate that HPV10

cells show intermediate ROS levels. These cells also represent an intermediate stage (localized cancer) between RWPE1 and PC3 cells. Kumar et al. [55] also demonstrated that ROS, mainly generated by the Nox system (trans-membrane proteins called the NADPH oxidases), is essential for deregulated growth, colony formation, cell migration and invasion and contribute to tumour metastasization. Besides the increase of ROS levels in PC3 cells, we found lower levels of MDA and higher levels of TAS that are consistent with cell resistance to OS. Therefore, these results suggest that PC3 cells counteract OS by expressing particular free radical scavengers. In fact, we found significant increase of GSH and Gl-Red activity in PC3 that may protect these cells under persistent ROS (peroxides) production, induced by radiotherapy and chemotherapy. H<sub>2</sub>O<sub>2</sub> treatment induced a clear decrease in TAS that is in agreement with the increase of MDA. These observations may be due to the absence of a



significant increase in the antioxidant defences, in face of the  $\text{H}_2\text{O}_2$  insult. On the other hand it may be explained by the high basal levels of GSH content and Gl-Red activity observed in PC3. However, we may consider other explanations for the observed decrease of TAS and increase of MDA. Namely, as TAS evaluation is based on the antioxidants capacity to inhibit  $\text{ABTS}^+$  radical cation (2,2'-azino-di-[3-ethylbenzotiazolin sulphonate]) [42] and that the antioxidant system may be recruited to face the increase of  $\text{H}_2\text{O}_2$ , we admit that the antioxidant defences are unavailable to inhibit  $\text{ABTS}^+$ , leading to TAS reduction observations. Moreover, the antioxidant system comprises other antioxidant defences like catalase, playing an important role in neutralizing  $\text{H}_2\text{O}_2$ , or superoxide dismutase and  $\alpha$ -tocopherol (that protects from lipid peroxidation), which we did not analysed and may also contribute to explain the decrease of TAS and increase of MDA.

In contrast, Gl-Px activity and GST are depleted in PC3 suggesting that GST depletion may facilitate prostate cancer progression as described by others [8]. The same assumption may be addressed to Gl-Px activity.

GSH, the major thiol in mammalian cells, maintains an optimum cellular redox potential through the inactivation of  $\text{H}_2\text{O}_2$ . The contribution of GSH and Gl-Red in PC3 cells protection against ROS was also suggested by Lim et al. [9]. These authors compared two metastatic prostate cancer cells, LNCaP, and PC3 and found lower ROS levels in PC3 that could be explained by an increase of GSH content, Gl-Red, thioredoxin reductase (TR) and GST activities. However, Kumar et al. [55] contradict those results showing higher ROS levels in PC3 comparing to LNCaP. In our study, PC3 Gl-Red activity is in the range values found by Jung et al. [3]. These authors also reported an increase in Gl-Red and a decrease in GST and Gl-Px activities in metastatic prostate cancer cells (PC3, LNCaP and DU145) comparing with primary cell cultures of benign and malignant human prostatic tissue. They also show higher Gl-Px and lower Gl-Red activities in PC3 cells comparing to other metastatic cell lines, reinforcing our observations that Gl-Px is decreased and Gl-Red is increased in advanced tumours cells. Therefore Gl-Red may contribute to protection against ROS.

To confirm the role of GSH content and Gl-Red activity in the adaptation to OS by metastatic prostate cancer cells, we treat the cells with DEM, a thiol depletion agent. Our study revealed that cells treated with 50  $\mu\text{M}$  DEM alone show a decrease in Gl-Red activity, additionally to a decrease in GSH content (Fig. 7A1) with no interference on cell proliferation and death. However, in the presence of  $\text{H}_2\text{O}_2$  the decrease in Gl-Red activity and GSH content was accomplished by a decrease in cell proliferation and increase of cell death preferentially by apoptosis and late apoptosis/necrosis. GSH reduction in the presence of DEM is in agreement with the results obtained by Coffey et al. [59,60]. These authors show an increase of apoptosis induced by radiation, in the presence of DEM, in PC3 and in other metastatic prostate cancer cells, LNCaP and DU145. They also found that apoptosis is accompanied by an efflux of GSH from the nucleus to the cytosol and admit that the presence of GSH in the nucleus may offer resistance to apoptosis.

Here we strongly proved that Gl-Red activity reduction participates in the increase of  $\text{H}_2\text{O}_2$  toxicity in metastatic prostate cancer cells, suggesting a potential therapeutic approach. It may be also related with GSH reduction as observed here and by others [59,60]. These results also conduct to the possibility of using other Gl-Red activity reducing agents or to evaluate the combined effect of DEM with conventional chemotherapeutic drugs, namely docetaxel, expecting that the association may allow to lowering drug concentrations, therefore reducing drug side effects, related with systemic toxicity. In fact we previously found that sodium selenite, a thiol depleting agent, combined with docetaxel, play a synergistic effect on PC3 cells growth inhibition and induces cell death [38]. Likewise our study warrants further evaluation of OS modulation

in prostate cancer therapeutic approach. In particular, it would be interesting to evaluate the effect of DEM-produced GSH depletion and Gl-Red reduced activity in the RWPE cells, in order to evaluate the potential toxicity in non-tumour cells.

## 5. Conclusions

As described above, other reports have compared the relative efficacy of antioxidant system in prostate cancer. However, this work develops a more graded study by including models representative of different stages of prostate malignancy.

To our knowledge, our study performed on cell lines representing normal prostate epithelium, localized and metastatic prostate cancer, all stages of prostate cancer progression, is the first demonstrating an increase of ROS (peroxides) along with prostate cancer progression, concomitantly with OS adaptation. Therefore we suggest that this duality may be necessary for prostate cancer progression and for a more aggressive malignant phenotype. On the other hand, as normal epithelium cell line shows lower ROS levels in basal conditions, but greater sensitivity to cytotoxicity induced by ROS (500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ) and concomitantly lower GSH levels and Gl-Red activity, these conditions may contribute to a higher susceptibility to OS lesion by normal prostate epithelium.

The cell line model, presented here shows an important approach to understanding OS through the different stages of prostate cancer. However, it is possible that cell lines do not exactly reflect the true in vivo situation. We also realize that the present strategy, developed in a cell line model, may not be efficient when applied to in vivo experiments. Different culture conditions could influence the observed results. However, this study does propose OS modulation as a possible new therapeutic approach in prostate cancer. We intend to carry out further evaluation of this strategy to in vivo model.

Furthermore, as far as we are aware, we are the first to confirm the pivotal role of Gl-Red's activity as a new target-directed therapeutic tool in the treatment of prostate cancer.

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